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# SHAKING PALSY:

# THE ROLE OF FREE RADICALS IN THE PATHOPHYSIOLOGY OF PARKINSON'S DISEASE

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ABBREVIATIONS:

AIF- apoptosis inducing factor; AGE- advanced glycation end-products; BMAA- B-methylamino-L-alanine; BDNF- brain-derived neurotrophic factor; CNS-central nervous system; COMT- catechol-O-methyl-transferase; CSF- cerebrospinal fluid; ELISA- enzyme-linked immunosorbent assay; GABA- γ-amino-butiric acid; GDNF- glial cell line-derived neurotrophic factor; GPx- glutathione peroxidase; GSH-glutathione; iNOS- inducible nitric oxide synthase; MAO-A - monoamine oxidase A; MAO-B- monoamine oxidase B; $MPP^+$- 1-methyl-4-phenylpyridinium; MPTP- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mtDNA-mitochondrial DNA;NMDA- N-methyl-D-aspartate; 6-OHDA- 6- hydroxydopamine; PARS- poly-ADP ribose polymerase; PD-Parkinson's disease; PUFA- polyunsaturated fatty acids; PTP- permeability transition pore; ROS- reactive oxygen species; SN- substantia nigra; SNc-substantia nigra pars compacta; SOD- superoxide dismutase; TaClo- 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline; TBA- thiobarbituric acid; TCA-tricarboxylic acids; TUNEL- terminal deoxynucleotidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling; TNF- tumor necrosis factor

## TABLE OF CONTENTS



## 1. Abstract

Parkinson's disease is a chronic illness classified within the category of neurodegenative diseases along with Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, Friedreich ataxia and others. It consists of progressive degeneration of the dopaminergic neurons in the substantia nigra in the midbrain. Its onset was correlated with aging, toxic events, and metabolic causes. There is no clear-cut pattern of inheritance in Parkinson's disease, although a genetic component was incriminated in a small percentage of cases. There is strong evidence that it may be caused largely by oxidative stress and metal toxicity; patients have low levels of natural antioxidants (glutathione and superoxide dismutase) and high levels of iron in the substantia nigra and other areas of their brain, leading to the hypothesis that iron and other transition metals help catalyze the free radical reactions that destroy the dopaminergic neurons, and that high levels of antioxidants (e.g., uric acid, flavonoids, ubiquinone) may help prevent or slow down the progress of Parkinson's disease. This review paper is dedicated to analyzing this hypothesis and also attempts to define possible future directions of investigation.

## 2. Introduction

Parkinson's disease is a chronic illness due to the progressive degeneration of those neurons that normally produce the neurotransmitter dopamine, leading to a dopamine deficiency in the basal ganglia. It is characterized by the following symptoms: muscular rigidity, resting tremor, bradykinesia (slow movement) or akinesia (absence or difficulty in initiating movement), shuffling gait, and stooped posture; depression or dementia may also be present at later stages. The most characteristic symptom, the accentuated tremor, led the British physician who first described this disease in 1817, James Parkinson, to name it "shaking palsy". Parkinson's disease is more common among men than among women and is four times more frequent in Caucasians than in darkly pigmented races. In the United States, PD affects about 1% of the population over age 60 [1]. Parkinson's disease is idiopathic, *i.e.* of no known cause. It is rarely inherited and less than 1% of all cases are believed to have a genetic component. The main pathological feature of PD is the progressive destruction of dopamine-producing cells in the substantia nigra region of the brain stem. The loss of dopamine affects the balance between dopamine and acetylcholine in the brain and subsequently the output to various muscles. Symptoms develop once 70% of the dopaminergic neurons in the substantia nigra have been destroyed [1].

Dopamine replacement is still considered the most efficient treatment for PD. Dopamine itself does not cross the blood-brain barrier and is consequently administered in the form of its chemical precursor, levodopa. Levodopa is given in combination with carbidopa, which inhibits the metabolism of levodopa in peripheral tissues in order to maximize its CNS effect. Dopamine agonists (*e.g.* bromocriptine) are also used. Avaiable surgical techniques are either ablative (thalamotomy and pallidotomy), augmentative (high-frequency deep-brain stimulation) or restaurative (gene therapy and neurotransplantation). Additional adjunctive therapies include physical therapy and nutritional counseling. However, the treatment is not curative; it can only

relieve symptoms and slow the progression of the disease. There is no known treatment able to halt or reverse neuronal degeneration in PD; however, substantial scientific advancements have been made in the past years towards the understanding of this disease [1].

The remainder of this review paper is devoted to a brief anatomical background and to the analysis of the current hypothesis of the international research community stating that free radical toxicity is involved in the pathophysiology of Parkinson's disease.

## 2. Pathological anatomy

The basal ganglia are a conglomeration of five distinguishable interconnected nuclei located at the base of the brain: the globus pallidus, the subthalamic nucleus, the striatum (consisting of caudate nucleus and putamen) and the substantia nigra. The basal ganglia became known as a component of the so-called extrapyramidal system, which is interconnected and cooperates with the pyramidal (corticospinal) system. The basal ganglia play an important role in voluntary movement and also have a role in cognitive function [2].

The substantia nigra is a pigmented mass of neurons located in the midbrain (mesencephalon) between the cerebral peduncles and the tegmentum. It is composed of a dorsal pars compacta containing melanin pigment, a ventral pars reticulata containing iron compounds, and a pars lateralis. The dendrites of neurons in the pars compacta arborize in the pars reticulata. The neuronal population of the substantia nigra consists of pigmented and nonpigmented neurons. Pigmented neurons outnumber nonpigmented neurons two to one. The neurotransmitter in pigmented neurons is dopamine. Nonpigmented neurons are either cholinergic or GABAergic. There is a characteristic pattern of neuronal loss in the substantia nigra in different disease states. Both pigmented and nonpigmented neurons are lost in patients with Huntington's chorea, another neurodegenerative disease of basal ganglia origin, which differs from PD in that it is a genetic disease with autosomal dominant inheritance. Only

pigmented (dopaminergic) neurons are lost in PD. In idiopathic PD this loss occurs mostly in the center of the substantia nigra, while in the postencephalitic type of PD the pattern is uniform throughout the substantia nigra [2]. Besides the pars compacta of the substantia nigra, two other cell groups in the mesencephalic tegmentum are dopaminergic: the ventral tegmental area of Tsai (located in close proximity to the medial substantia nigra and usually spared in PD) and the retrorubral cell group (moderately affected in PD) [2].

In postmortem studies it was discovered that the substantia nigra (meaning "black substance") had lost its pigment in PD patients. The pigment is called neuromelanin and was commented on as long ago as 1786 by Vicq d'Azyr. Apart from the substantia nigra, neuromelanin is also located in the locus coeruleus in the pons, where the neurotransmitter is norepinephrine. It is certain, however, that neuromelanin is a recent phylogenetic development and it appears that the intensity of pigmentation is related to the degree of evolution of the brain. Neuromelanin is present only in the brain of humans, primates and some carnivores, *e.g.* the dog and the cat; it does not appear to be present in the brain of common laboratory animals, such as the rat, the guinea pig, and the rabbit. Evidence suggests that neuromelanin is not synthesized by a tyrosine-tyrosinase system as in skin melanocytes, but by a different pathway involving oxidation of brain catecholamines, in particular dopamine. Moreover, there is a striking similarity between the distribution of neuromelanin and that of the brain catecholamines – norepinephrine and dopamine [3]. These anatomical findings inevitably led to the conclusion that melanin in neurons is intimately related to the ability of these cells to synthesize dopamine or norepinephrine. Indeed, neuromelanin may be looked upon as an indication of catecholamine synthesis in neuronal systems containing dopamine or norepinephrine. The question arises as to why melanin in neurons containing catecholamines should be practically confined to primates and carnivores. A possibility is that the appearance of melanin in catecholamine-containing cells

is the outcome of long-continued amine synthesis and depends on the life span of the species [3]. Neuromelanin has a strict anatomical localization and a late evolutionary appearance which suggest that it serves some positive function. Melanin deposition may provide a mechanism of inactivation of some metabolic product possibly of catecholamine origin which cannot be removed from neurons by other means. Alternatively, the chemical constituents of melanin, in particular the heavy metals it contains – zinc, copper, iron, manganese, titanium, cobalt, and nickel – may influence neuronal metabolism. A significant feature of neuromelanin is the presence of free semiquinone radicals, which might influence oxidation-reduction reactions [3].

Parkinson's disease affects the cytoskeleton and damage occurs only in selectively vulnerable neurons. As a result of the cytoskeletal changes, the so-called Lewy bodies may appear; their major components are abnormally phosphorylated neurofilaments, in other words altered "building blocks" of the cytoskeleton. The Lewy bodies are concentric hyaline cytoplasmic inclusions seen in pigmented nigral cells, but not in all cases of PD (e.g., not in those cases following encephalitis letargica) [3].

## 3. Physiopathology

Parkinsonism may occur following carbon monoxide poisoning or cranial trauma ( *e.g.* dementia pugilistica, a condition seen in professional boxers such as Muhammad Ali). Parkinson's disease may also develop later in life, in connection with a neurotoxic event that occurred at an early age, *e.g.* encephalitis lethargica or a whooping cough *(pertussis*) epidemic infection. Certain diuretics (*e.g.* reserpine), antipsychotics (*e.g.*, chlorpromazine), non-steroidal antiinflamatory drugs (e.g. naproxen), calcium channel blockers (*e.g.* verapamil) and "designer drugs" (*e.g.* MPTP) have all been implicated in causing or exacerbating PD. Environmental and dietary factors may also be involved in the pathogenesis of this disease, *i.e.* occupational exposure to herbicides and pesticides, the high intake of animal fats, the high intake of sugars

(mono- and disaccharides), the high aluminum content of drinking water, the mercury from dental amalgams, the presence of other metals, notably manganese, cadmium and copper, as well as cerebrovascular disease (*i.e.* multiple lacunar strokes) [4].

A consensus is now emerging that major causes of neurodegenerative diseases in general, and PD in particular, are oxidative stress (*i.e.*, an excess of free radicals) and metal toxicity [5]. The following section of this paper is devoted to the critical review of this hypothesis. In relation to this hypothesis, one of the above-mentioned causative factors, MPTP, is entitled to a special discussion. MPTP-induced parkinsonism is a model for PD in human and non-human primates and was discovered accidentally. In 1982, several heroin addicts in California and Maryland suddenly developed acute and permanent parkinsonism after injecting a synthetic opiate containing the impurity MPTP. The symptoms required constant levodopa treatment ever since. MPTP is converted by the neuronal enzyme MAO-B into its toxic metabolite $MPP^+$ which has structural similarities to dopamine and is taken up into the dopaminergic nerve terminal by the dopamine uptake system where it causes cell damage. Another drug, selegiline (or deprenyl), an almost forgotten substance that was invented by a Hungarian scientist and failed its purpose as an antidepressant, was found to have antagonist actions to MPTP since it is a selective and irreversible inhibitor of MAO-B, preventing dopamine depletion and PD symptoms [6]. Several mechanisms of toxicity, further addressed in this paper, have been proposed to explain the actions of MPTP.

## 5. Theories of free radical involvement in Parkinson's disease

One of the leading hypotheses nowadays is that oxidative stress by ROS damages essential components of the dopaminergic neurons in the SNc, including DNA, protein structures and the cell membrane, resulting in functional disruption and ultimate cell death. These free radicals are produced during normal cellular metabolism, but specific cellular detoxification systems

normally minimize their damaging effects [7]. Although ROS level cannot be measured directly in the living patient, post-mortem studies of the brain in PD have revealed:

1) increased membrane peroxidation in the SNc and widespread ROS-induced protein modification, with protein carbonyl levels elevated in all regions of the brain [7,8];

2) elevated TBA-reactive substance levels (a measure of the secondary products of lipid peroxidation) accompanied by decreased PUFA levels (the peroxidation substrate) [8];

3) increased 8-hydroxy-2′deoxyguanosine levels in the SNc and other brain regions, indicative of ROS-mediated DNA damage [7,9].

Furthermore, the measured activities of a number of phospholipid-catabolizing enzymes in the normal SN have been found to be low compared with other regions of the human brain, suggesting slow phospholipid turnover in the SN, which would restrict the capacity for rapid repair of oxidative membrane damage [10].

There are several potential metabolic sources of increased oxidative stress in PD: dopamine metabolism, mitochondrial dysfunction, increased free iron levels, accumulation of AGEs, reduced activity of free radical defense systems, and the deleterious role of glial cells. Several toxins may also be implicated in PD. A brief discussion is devoted to each of these hypotheses.

*1) Dopamine metabolism*

Dopamine is oxidized in the brain *via* two pathways.

a) Auto-oxidation of dopamine leads to the production of toxic semiquinone species ($SQ^{\bullet}$), which *via* a metabolic cascade can be polymerized to neuromelanin. Little can be said with certainty about the effect of neuromelanin on neuronal function. It is generally regarded as a waste product of dopamine auto-oxidation and as a “double-edged sword” with respect to free radical reactions, in that it sequesters redox-active metal ions (such as iron), but may also

promote ROS-generating processes and may also represent a depot for other cellular toxins, such as $MPP^+$ [11].

b) In biochemical catalysis, primary, secondary and tertiary amines, including the neuronal transmitters or hormones dopamine, noradrenaline, adrenaline and serotonin, are broken down into their respective metabolites in a two-step enzymatic conversion. The general equation is:

$$\text{Monoamine} + H_2O + O_2 \rightarrow \text{Aldehyde} + NH_3 + H_2O_2 \quad (1)$$

$$\text{Aldehyde} + \text{NAD(P)} + H_2O \rightarrow \text{Acid} + \text{NAD(P)H} + H^+ \quad (2)$$

In this particular case, enzymatic metabolism of dopamine by MAO generates $H_2O_2$ which is normally inactivated by GPx and its cofactor GSH. If dopamine turnover were increased during the preclinical phase of PD as part of a compensation effort, and if the GSH system itself were deficient, excessive $H_2O_2$ production and its conversion to $^{\bullet}OH$ *via* the iron-mediated Fenton reaction (3) could generate increased local oxidative stress [7]:

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \quad (3)$$

Also, MAO-B, but not MAO-A, is found at an increased level (approximately 25% higher) in the SN of PD patients; MAO-B in blood platelets is significantly higher in PD and Alzheimer's disease [6]. This data points out the role of MAO-B in accelerating dopamine breakdown in PD pathogenesis. It is worth mentioning that, *in vitro*, $H_2O_2$ induces the activity of MAO-B but not that of MAO-A [6]. If the same action would be confirmed *in vivo*, then it would be worth considering a feedback-positive mechanism in which dopamine oxidation, catalyzed by MAO-B, produces $H_2O_2$ that would only further enhance the activity of MAO-B.

*2) Mitochondrial dysfunction*

Evidence implying that mitochondria have a crucial role in both necrotic and apoptotic cell death is accumulating rapidly. Both these conditions are distinctive forms of cell death, as defined morphologically; however, in neuronal populations and diseases such as PD they can

either coexist or be sequential events. Fig. 1 (from [12]) shows the involvement of mitochondria in cell death. Fig. 1a depicts a severe insult resulting in death by necrosis (*via* prominent and persistent depolarization of the mitochondrial membrane potential followed by a depletion of energy) while Fig. 1b depicts a mild insult resulting in apoptosis (*via* initiation of the caspase cascade and disruption of oxidative phosphorylation). The nature of neuronal death in Parkinson's disease is still undetermined, but there are indications that it involves a combination of apoptotic processes and necrotic degeneration. It is possible that low level exposure to a toxin or oxidative stress initially stimulates apoptotic losses, but with prolonged exposure can also induce necrotic neurodegeneration [7].

The CNS has a particularly high energy requirement, thus making it very susceptible to defects in mitochondrial function A consequence of mitochondrial dysfunction is increased generation of free radicals and oxidative damage, which are strongly implied in the pathogenesis of neurodegenerative diseases. Mitochondria, the most important physiological sourse of $O_2^{\bullet}$ in animal cells, are estimated to produce 2-3 nanomoles of $O_2^{\bullet}$/min/mg protein [12].

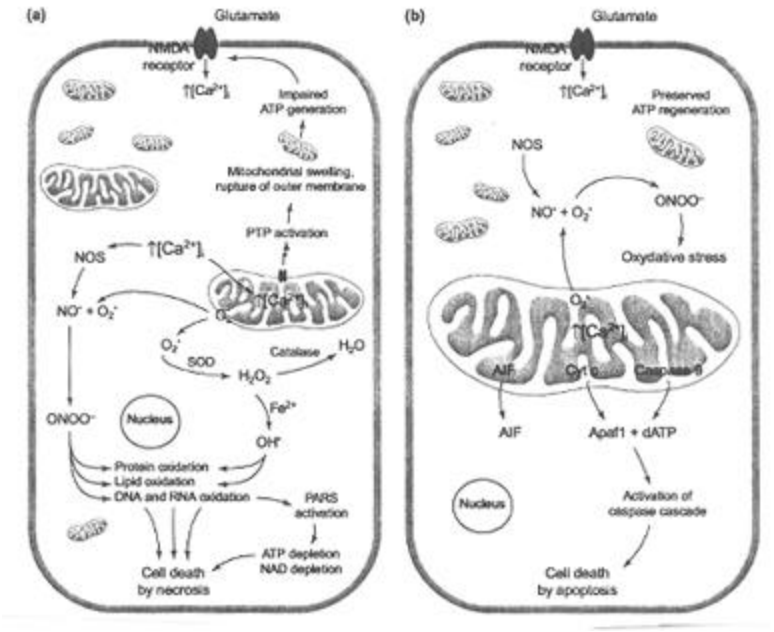


Fig. 1. Involvement of mitochondria in cell death

One of the known substrates of mitochondrial dysfunction in PD consists of abnormalities of the respiratory chain (schematically drawn in Fig.2, from [13]). The respiratory chain consists of five complexes aligned along the inner membrane of the mitochondria. The functions of the respiratory chain are proton pumping, electron transport, conversion of atomic oxygen into water, and, most importantly, production of energy in the form of ATP. In total, the five enzyme complexes are formed by more than 80 subunits; 13 of these are encoded by the mitochondrial genome and the others by nuclear DNA. In MPTP-induced parkinsonism, MPTP is metabolized to $MPP^+$ which is preferentially stored in the mitochondria of dopaminergic neurons in the SN, where it inhibits complex I of the electron-transport chain, leading to reductions in ATP production and elevated ROS generation, which in turn result in oxidative damage to various cell structures [7]. Whether this action accounts entirely for the toxic potential of MPTP is still unclear, but there is significant evidence supporting the hypothesis that progressive reduction in mitochondrial respiration (a feature of the aging brain) is involved in a number of neurodegenerative diseases, including PD [12]. In idiopathic PD there is a 30-40% decrease in complex I activity in the SN [12, 14] and reduced staining for complex I subunits in the SN, although preserved staining for subunits of other electron-transport complexes, as demonstrated immunohistochemically [15].

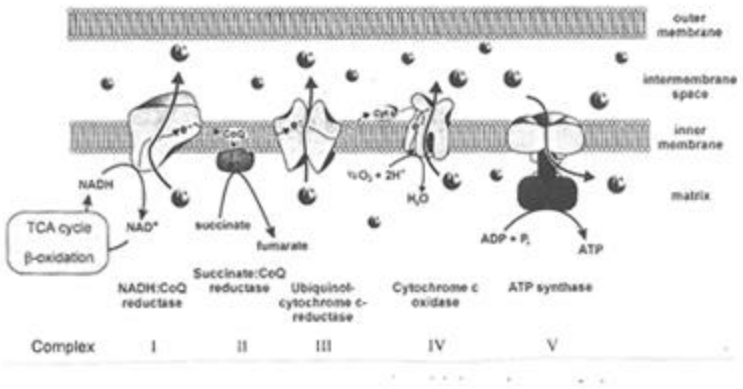


Fig. 2. Schematic drawing of the mitochondrial respiratory chain

An useful technique for investigating the role of mtDNA defects in neurodegenerative diseases, pioneered by King and Attardi, is the production of cybrid cell lines. Cybrids are cells formed by fusing mitochondria from a patient's platelets or other tissues with *rho*$^0$ cells that are mtDNA-deficient cell lines. Cells can be depleted of mtDNA by exposing them to low concentrations of ethidium bromide, which inhibits replication of mitochondrial but not nuclear DNA. Exposed cells lose their mtDNA and assume an anaerobic phenotype. If defects in oxidative phosphorylation are found in the cybrids, they are attributable to a patient's mtDNA, as the patient's mitochondria now function in the presence of a different nuclear genome [12]. Strong support for a mtDNA-encoded defect comes from two studies proving that cybrids made from PD patients show reductions in complex I activity. These defects are associated with increased free radical production, increased susceptibility to $MPP^+$ and impaired mitochondrial $Ca^{2+}$ buffering [12, 16]. It is now generally accepted that PD patients present a complex I defect in their substantia nigra. Some authors have argued that in PD complex I deficiency is systemic, after having had detected this feature in several peripheric tissues, including platelets, thus offering the interesting possibility of a convenient biomarker for PD [17]. The question of whether this defect is primary or secondary (*i.e.*, the result of toxic, oxidative or other damage) is currently the focus of intensive research.

*3. Increased free iron levels*

The normal basal ganglia are rich in iron, with highest levels in the SN, globus pallidus and putamen. Absolute iron levels are increased in PD by about 35%, specifically in the SNc [18]. Of particular significance in PD is the shift in the Fe(II)/Fe(III) ratio from almost 2:1 to 1:2 [18], consistent with increased Fe(II)-catalyzed conversion of $H_2O_2$ to the highly reactive $^{\bullet}OH$ in the Fenton reaction [10]:

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + OH^{-} + Fe^{3+}$$

The reduction of Fe(III) by the superoxide radical would increase the rate of the Fenton reaction, thus driving the Haber-Weiss cycle-mediated production of $^{\bullet}OH$ (4) [7]:

$$O_2^{\bullet-} + H_2O_2 \rightarrow HO^{\bullet} + OH^{-} + O_2 \quad (4)$$

The availability of soluble iron complexes for participation in this reaction is a major determinant of the toxicity of ROS in the neuron. Iron participates in free radical-generating reactions only in the free, ferrous form. Ferric iron in the SNc is normally bound either by ferritin (about 90%) or by neuromelanin (10%). A variety of molecules with an *o*-dihydroxy-phenyl structure (including 6-OHDA and dopamine) effectively release ferritin-bound iron in vitro and thereby stimulate lipid peroxidation [7,19]; the iron chelator desferrioxamine has antagonistic actions, protecting rats against 6-OHDA induced loss of striatal dopamine [20]. In animal models, infusion of iron into the SNc induced neurodegeneration and a dose-related decline in striatal dopamine. In PD, it is not known whether the increased iron levels are due to reduced ferritin levels or increased cellular uptake of iron. It is considered unlikely that iron is involved early in the pathogenesis of PD, since laboratory tests have not indicated elevated levels of iron in the preclinical stage of the disease, however, its potent ability to generate ROS means that it may play a significant role in the progression of PD [7], especially as it is accompanied by a dramatic rise in aluminum levels [21] which would exacerbate iron-related oxidative damage; aluminum salts accelerate lipid peroxidation induced by iron salts [21].

In correlation with the chemistry of iron, it was shown that the enzyme SOD may be involved in many neurodegenerative diseases. Evidence comes from studies demonstrating that the inactivation of the mouse gene for mitochondrial superoxide dismutase leads to tissue-specific inhibition of the respiratory chain enzymes complex I and complex II, inactivation of the TCA cycle enzyme aconitase and accumulation of DNA oxidative damage [22]. Complex II contains

three Fe-S clusters, only one of which is tetranuclear (4Fe-4S) cluster. The inactivation of aconitase in SOD-deficient mice is likely to be caused by oxidation of its Fe-S cluster by superoxide, which liberates the iron as $Fe^{2+}$; this iron can then participate in the Fenton reaction, generating hydroxyl radical and leading to further oxidative damage [22]. A decrease in aconitase activity is seen in various neurodegenerative diseases, e.g. Friedreich ataxia, and is worth exploring in PD as well.

*4. Accumulation of AGEs*

Advanced glycation end-products are a group of substances formed by a cascade of reaction that follow the reaction of protein amino groups (especially the N-terminal and side chain amino groups of lysine and arginine) with monosaccharide molecules [23]. Their accumulation in the ageing human brain is facilitated by oxidative stress and transition metals, including iron, and their formation is inhibited by radical scavengers and thiol antioxidants. Highly reactive dicarbonyl products and ROS are produced in the course of AGE synthesis, thus further enhancing oxidative stress. Due to these properties and their promotion of protein cross-linking, AGEs have been implicated in degenerative disorders associated with ageing in the periphery as well as in the CNS [7]. In Alzheimer's disease, AGEs promote the polymerization of ß-amyloid, the major component of plaques [24], and may similarly be involved in PD in cross-linking underlying Lewy body formation [24]. In the SN of PD patients were identified two AGEs (pentosidine and pyralline) and heme-oxygenase-1, a putative marker for oxidative stress. Heme-oxygenase-1 immunoreactivity was localized to the filaments of Lewy bodies, suggesting a particular sensitivity of the cytoskeleton [24].

*5. Reduced activity of free radical defense systems*

The cellular radical detoxification system (its most important components being SOD and GPx) is supposed to neutralize free radicals by catalyzing reactions (5) and (6), respectively:

$$^{?}O_2 + {}^{?}O_2 + 2H^{+} \; ? \quad H_2O_2 + O_2 \qquad (5)$$

$$2GSH + H_2O_2 \; ? \quad GSSG + 2H_2O \qquad (6)$$

However, the activity of this system declines, even with normal aging, and, even in normal individuals, the neurons in the SNc are particularly vulnerable enzymatically. In the normal brain, nigral GSH levels are low in comparison with other brain regions [25]. In PD, there is a further decline in GSH levels without an increase in the oxidized form, GSSG, which suggests a decrease in GPx activity in the SN. Of the other enzymes which modulate GSH levels, only the levels of ?-glutamyl transpeptidase, which is associated with GSH translocation and degradation, are significantly different (doubled). This change is restricted to PD and the SNc [25]. It seems that the decline in GSH levels occurs early in the course of the disorder, and hence the view that GSH depletion represents a "first blow" that renders the cell vulnerable to neurodegeneration possibly induced by other factors [25].

Melatonin, the hormone secreted by the pineal gland (epiphysis) and involved in the regulation of the circadian rhythm, was recently reported to have antioxidant and anti-apoptotic properties in animal and cell culture models: free radical scavenging, up-regulation of several antioxidant enzymes (including SOD and GPx) and inhibition of NOS [26]. Endogenous melatonin levels decline with age, and this change may increase the vulnerability of susceptible regions to oxidative damage.

*6. The deleterious actions of glial cells*

The debate focusing on the role of glial cells in PD is very interesting. Until recently, it was assumed that glial cells have a protective role, since glial cell distribution is not uniform across the brainstem: it is lowest in the areas that are prone to PD and highest in the areas where dopaminergic neurons are preserved [27]. The mechanisms of neuroprotection by the glial cells could consist of secretion of trophic factors or the high degree of expression of GPx [27].

However, it was suggested recently that glial cells may damage dopaminergic neurons by secreting factors that might diffuse into neurons or act on membrane receptors. The density of glial cells expressing pro-inflammatory cytokines such as TNF-a, interferon ? and interleukine 1ß is increased in the SN in PD [28]. Cytokines were found to induce the expression of CD23, a low-affinity immunoglobulin E receptor which, following appropriate ligation, induces the expression of the enzyme NOS II that produces nitric oxide which is diffusible and may penetrate into neurons, combine with superoxide radicals and form peroxinitrates that are extremely toxic; indeed, the concentration of nitrites is increased in the CSF of PD patients [29], and 3-nitrotyrosine, an index of protein nitrosation induced by peroxinitrate, was detected in nigral dopaminergic neurons in PD [30].

CSF in PD was reported to have cytotoxic effects that are specific for dopaminergic neurons and mediated by TNF-a [31]. The TNF-a levels in CSF were determined by ELISA. The mean TNF-a level was 2.6-fold higher in CSF specimens from PD patients than in those of controls. It was shown that CSF specimens obtained by lombar puncture from these patients were cytotoxic to dopaminergic neurons in primary mesencephalic cell culture. Specimens of CSF were evaluated in dopaminergic and non-dopaminergic cell lines for cytotoxicity by viability assay and by the inhibition of tyrosine hydroxylase. Cytotoxicity was assessed by cell structure and cell viability staining. To explore the possible involvement of apoptotic mechanisms in CSF-induced cell injury, these cells were also stained for DNA fragmentation with the TUNEL technique. A typical pattern that included nuclear chromatin condensation and nuclear fragmentation was apparent after incubation with CSF, suggesting the role of apoptotic mechanisms. Along with TNF-a, interleukin 1ß was also elevated in CSF in PD [31].

A summary of the potential deleterious or neuroprotective actions of glial cells is shown in Figure 3 (from [27]):

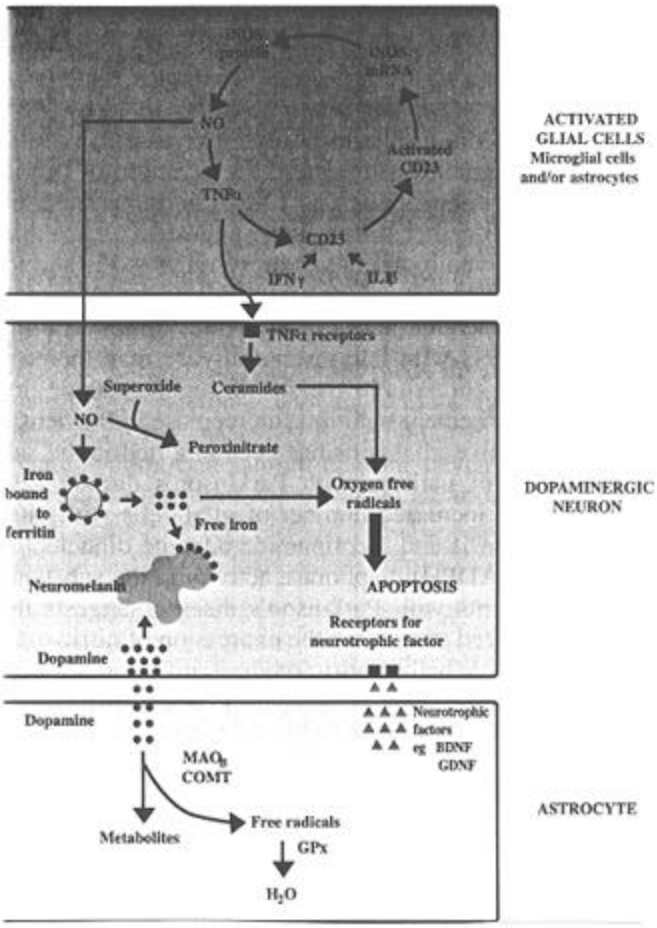


Fig. 3. The potential deleterious and neuroprotective effects of glial cells

*7) The generation of free radicals by toxins in PD*

The recognition that the inhibition of mitochondrial complex I by $MPP^+$ (and also by paraquat and 6-OHDA) not only leads to energy deficits but also the generation of $O_2^{?-}$, $^{?}OH$ and $H_2O_2$, and thus possibly to oxidative stress, stimulated the search for a "natural" inhibitor that might represent the causative agent in PD [7]. The possibility that an external toxin is involved was suggerated by epidemiological studies which found a greater risk for PD associated with exposure to any of a range of seemingly unrelated variables: agricultural chemicals, industrial environments, different metals. The development of multifactorial or interaction models may allow the detection of at-risk individuals without first identifying a specific toxin [7].

A summary of the cellular mechanisms which may be involved in the neurodegenerative process in PD is shown in Figure 4 (from [7]) in an attempt to develop such an interaction model, taking into consideration the dynamics of all the factors shown above:

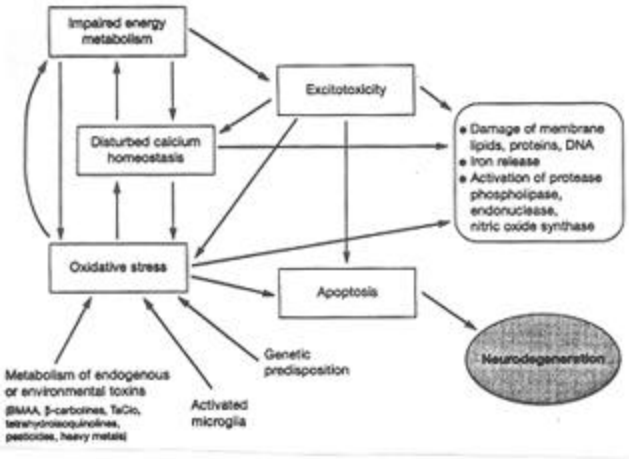


Fig. 4. Cellular mechanisms which may be involved in neurodegeneration in Parkinson's disease

## 6. Suggested experimental approaches in Parkinson's disease

One of the reviews quoted in this paper is the one signed by Hans Larsen: *Parkinson's Disease: Is Victory in Sight?* Indeed, nowadays, almost two centuries after the first written description recorded of this disease, *Essay on the Shaking Palsy* by James Parkinson, it is a challenge to engage in this research field, considering that there is, in fact, no known treatment able to halt or reverse the process of degeneration of dopaminergic neurons. The hope for a cure is still alive, and a cure will be eventually found; however, it is remarkable that, with practically no clinical approach left untried, the best is yet to come in Parkinson's disease therapy. As far as we know, neurodegeneration- whether slower or faster- is irreversible. Every single method that has been tried until now has its own limitations, and none is universal. Responsiveness to levodopa decreases gradually; immunotherapy has failed; graft survival in neurotransplantation is variable and restricted by the number of human fetal donors avaiable; xenotransplantation is subject to acute vascular rejection after a few days; thalamotomy abolishes tremor only

unilaterally; gene therapy is by no means performed on a large scale, and in none of the above was a full recovery ever recorded. The answer is probably to be found in the title of another article, that of M. Piccolino: *Old Cells, Old Experiments, New Results.* The critical assimilation of the literature does not lead to the belief that the cure for Parkinson's disease will consist in a "magic bullet", as penicillin was for tuberculosis in the the time of Alexander Fleming. It is more likely that, starting from the existing accomplishments of the contemporary research scene, several main directions of future investigation will emerge.

A scientist true to the classical conception of the anatomical school would indeed choose to pursue the hard facts: that only pigmented neurons degenerate in Parkinson's disease, and will focus on neuromelanin as an indication of catecholamine synthesis or a side-product of dopamine auto-oxidation in neurons. The properties of neuromelanin will certainly have to be investigated in detail, since its chemical identity with oculocutaneous melanin has never been established. Valuable starting points are the facts that: 1) unlike skin and eye melanin, neuromelanin is localized in neurons and not in melanocytes; 2) unlike skin and eye melanin, neuromelanin is present (spared) in the genetic disease albinism – an autosomal recessive disorder due to a deficiency in the activity of the enzyme tyrosinase; 3) the skin pigment seems to be a protective factor in some races against neurodegeneration, a cutaneous barrier that would bind toxins as neuromelanin binds $MPP^+$; 4) primary malignant melanomas of the CNS are not recorded; 4) neuromelanin binds and accumulates heavy metals. Regarding the comparison with skin melanin, it is significant that pigmented cells called melanophores in heterothermic vertebrates (*e.g.* amphibians) possess dopamine receptors that mediate melanosome aggregation and melonosome dispersion, and also receptors for norepinephrine [33]. Thus the ligand-receptor type of interaction between dopamine and melanin could be further explored at epithelial and neural level; on the basis of dose-response curves, a characterization of these receptors could be

attempted as a function of agonist affinity; the goal of such a research project would be the identification of several classes of receptors based on differences in their expression. The histochemical and spectroscopic properties of neuromelanin will have to be studied in detail, since the anatomical distribution, the biochemical pathways and the selective vulnerability to various diseases do not argue in favor of their chemical identity. A logical starting point would be the examination of the spatial structures of these biopolymers generally called melanins by X-ray diffraction techniques in a synchrotron radiation facility, by following changes in diffraction data during polymerization or depolymerization of melanins. This may help understanding the structural changes at different levels of organization in melanins. Also, since neuromelanin contains small amounts of metals, the immediate chemical environment of the heavy atom in the melanin structure and the location of the binding sites may also be worth investigating, and the most appropriate technique would probably be EXAFS (extended X-ray absorption fine structure). The principle of EXAFS is that when X-rays traverse a sample under study, the transmitted beam suffers an attenuation which normally changes monotonically; if the sample contains a heavy atom, the attenuation increase steeply. Conventional techniques as Perls staining and transmission electron microscopy have failed to properly serve the purposes of identifying neuromelanin granules in brain slices, but a promising technique is LAMMA (laser microprobe mass analyzer), an advanced type of mass spectrophotometer; the recording of peaks corresponds to increased quantities of iron and aluminum that may exist in Parkinson's disease and Alzheimer's disease. Finally, it is important to note that neuromelanin is believed to coexist in granules with lipofuscin, and that the nature of their association is at present very poorly understood; a starting point for future directions of research would be the model system in which bleaching with hydrogen peroxide gradually converts the optical and histochemical properties of melanin to those of lipofuscin and the reverse, in which lipofuscin, experimentally melanized

with catecholamines, acquires characteristic optical properties of neuromelanin [34]. Further investigation of this concept have materialized up to now in quantification of fluorescence intensity *versus* wavelength; future studies would help further characterize these spectra.

From the point of view of the physiologist, however, the most important issue would be function, and not structure. Recent studies have expanded the abnormalities of dopaminergic neurons well beyond the limits of the substantia nigra, and have challenged the conservative view that this is exclusively a motor system disorder. In PD, abnormal visually evoked potentials led to the conclusion that visual dysfunction is a result of dopaminergic deficiency in both humans and animals [35]. Pathology, especially the toxic metabolite $MPP^+$, may alter the balance between dopamine receptors. Dopamine might act in the retina by decoupling electrotonic junctions of horizontal cells involved in areal summation of photoreceptor signals. Light stimulates retinal tyrosine hydroxylase leading to the release of dopamine. Dopamine has a role in visual adaptation and in the center-surround organization of retinal receptive fields in various mammalian species. Obviously, neurophysiological techniques using intracellular recordings would be the best method of pursuing the issue of the broader functions of dopaminergic neurons affected in PD.

There is also evidence of an endogenous retinal clock regulating photoreceptor sensitivity [36]. Recent data suggest circadian control of contrast sensitivity *via* dopaminergic mechanisms in PD. How and where the circadian regulator interacts with endogenous relase and uptake of dopamine in the visual pathway is not known. As a physiology student, the author of this paper would attempt to suggest a correlation between the role of melatonin as a regulator of the circadian rhythm and this retinal clock, as endogenous melatonin levels decline with age and its role may be partly overtaken, in order to maintain the usual sleep-wakefulness cycle, by other systems in the body, notably the dopaminergic system of neural transmission; when suffering of

PD, this might affect elderly individuals. It is also very important to stress that parkinsonian tremor disappears almost completely during sleep.

It also may be that a relationship exists between retinal visual defects and cognitive visual dysfunction. PD patients have, indeed, deficits in performing orientation-dependent visual tasks. However, retinal neurons do not show orientation selectivity; these findings would suggest cortical defects. One possible reason may be that ascending dopamine deficiency may affect prefrontal circuits involved in the mnemonic coding of visual space. Another explanation would be that higher cortical structures are affected. These were indeed found to be the hippocampus (involved in short-term memory, which is the first type of memory to decline with age) and the brain amygdala, which influences autonomic and neurosecretory nuclei in the hypothalamus, and therefore the endocrine system. Neuroendocrine correlations, as was the previous one between melatonin and dopamine, would lead to completely novel physiological hypotheses.

A young immunologist would seek the revival of the abandoned autoimmune theory and would note that in PD the glial cells start phagocytizing neurons and express the HLA-DR antigen which is absent in controls; glial cells may signal to the periphery since they also express receptors for interleukin 2 which is secreted presumably only by T-cells [37]. A pharmacologist would probably have the strongest arguments for a research proposal. Antipsychotic medication in the form of neuroleptics prescribed for schizophrenia and mania (*e.g.*, chlorpromazine, haloperidol) act by blocking the dopamine receptors and induce side effects as tardive dyskinesia, depigmentation of the SN neurons and skin sensitivity [1]. It is now a fact that these drugs specifically bind melanins and this interaction may be analyzed *in vivo* by various methods, for example whole body autoradiography to study the distribution of radioactively labeled drugs in pigmented and albino mice. The double role of chlorpromazine as a free radical scavenger and a generator of toxic metabolites should also be taken into consideration.

## 7. Summary

PD is a progressive degeneration of dopaminergic neurons in the substantia nigra, leading to a disability of initiating voluntary movements. Treatment is at present only sympthomatic, and not curative, but the current research trends have provided a fascinating insight into its causes, which, whether toxic or metabolic, seem to have a common ground in free radical generation.

James Parkinson (1755-1824) first assisted, then took over his father's general practice in London. He became a surgeon, although he never attended college. He published two medical commentaries: one is the earliest known English text dealing with appendicitis with rupture, the other is *Essay on the Shaking Palsy*. He was also fascinated with geology and paleontology and wrote a three volume book about fossils entitled *Organic Remains of a Former World*. James Parkinson lived throughout the establishment of the United States, the French Revolution, the Napoleonic era and the industrial revolution. He was a social reformer, the author of pamphlets advocating universal suffrage, the removal of taxes on such necessities of life as soap, bread, and candles, a welfare system for the poor, the inalienable rights of the insane and the demented.

In 1623, another Englishman, a poet named John Donne, was writing:

"Perchance he for whom this bell tolls may be so ill, as that he knows not it tolls for him. This bell calls us all; but how much more me, who am brought so near the door by this sickness. No man is an island, entire of itself; every man is a piece of a continent, a part of the main. Any man's death diminishes me, because I am involved in mankind, and therefore never send to know for whom the bell tolls; it tolls for thee. Another man may be sick too, and sick to death, and this affliction may be of no use to him, but this bell tells me of his affliction; by consideration of another's danger I take mine own into contemplation. All mankind is of one author, and is one volume; when one man dies, one chapter is not torn out of the book, but

translated into a better language; and every chapter must be so translated. God employs several translators."

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